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(54) Production of L-amino acids by transamination.

(57) A process is described for producing alpha amino acids or derivatives thereof. The process comprises reacting an alpha-keto acid with L-aspartic acid in the presence of transaminase enzyme to produce (1) an alpha amino acid corresponding to said alpha-keto acid and (2) oxaloacetate; and decarboxylating said oxaloacetate.

Also decribed is a process for producing L-amino acids directly from the corresponding D,L-amino acid racemic mixture. The process comprises reacting said D,L-amino acid mixture in admixture with a D-amino acid oxidase and a transaminase in the presence of a suitable amino group donor.

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PRODUCTION OF L-AMINO ACIDS BY TRANSAMINATION

This invention relates to the production of alpha amino acids and derivatives thereof, and in particular, to the production of L-amino acids and their derivatives by transamination between L-aspartic acid and alpha-keto acid compounds corresponding to the desired amino acids. The invention also relates to the conversion of D, L-amino acids to L-amino acids in a single step process using D-amino acid oxidase to convert the D-amino acid to a 2-keto carboxylic acid and then using the transaminase to convert the 2-keto carboxylic acid to L-amino acid.

Background of the Invention

Amino acids currently have application as additives to animal feed, nutritional supplements for human food, components in infusion solutions, and synthetic intermediates for the manufacture of pharmaceuticals and agricultural chemicals. L-glutamic acid is used as a flavor enhancer for food with a world market of over 1 billion dollars annually. L-lysine and methionine are large volume additives to animal feed, and L-tryptophan and L-threonine have similar potential applications. L-phenylalanine and L-aspartic acid have very important markets as key components in the manufacture of the sweetener aspartate. Infusion solutions require a range of amino acids including those essential in human diets.

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Methods currently in use for the production of amino acids include extraction, chemical synthesis followed by resolution, fermentation and enzymatic synthesis (biocatalysis). The L-form of the amino acid is required for most applications but certain amino acids are much more easily produced in the racemic D,L form rather than the L-form. Extraction procedures require extensive purification of the amino acid of interest from protein hydrolyzates. With chemical synthetic methods, normally a racemic mixture is formed, and the resolution to produce the optically active product is often costly and inefficient. Fermentation, while overcoming many of the disadvantages inherent in the previously mentioned methods, suffers from problems of slow rates of conversion, dilute solutions, costly purifications, and very high capital costs. Biocatalysis offers the potential for lower cost production in many cases primarily because of the significantly reduced capital requirements, lower purification costs due to the absence byproducts in the product stream, and higher rates of conversion of substrates to products because fewer enzymatic steps are involved.

Some biocatalytic processes are currently in use. For example, L-aspartic acid is produced in commercial quantities by the reaction of fumaric acid with ammonia in the presence of the enzyme aspartase. See Tosa et al, Appl. Microbiol. 27, 886-9 (1974). L-phenylalanine can be produced by enzymatic synthesis from cinnamic acid and ammonia using the enzyme phenylalanine-ammonia lyase. L-alanine can be synthesized from

L-aspartic acid by enzymatic decarboxylation. See U.S. Patent Nos. 3,458,400 and 3,463,704. These processes are useful for the production of the individual amino acids listed. However, none of these processes is based on a general enzymatic technology broadly applicable to the production of many amino acids.

One enzymatic process broadly applicable to the production of many amino acids is described in U.S. Patent No. 3,183,170. In the process of U.S. 3,183,170 L-glutamic acid and a keto acid are combined with transaminase to produce alpha-keto glutaric acid and L-amino acid. The alpha-keto glutaric acid is continuously reduced to L-glutamic acid in the presence of a multi enzyme system, hydrogen gas, and a nitrogen source, such as inorganic ammonium salt, organic ammonium salt, ammonium hydroxide, ammonia gas, or urea. The L-glutamic acid thus produced is recycled thus enabling the production of large amounts of L-amino acid with a small quantity of L-glutamic acid. However, this multienzyme system is difficult to operate on a commercial scale because it requires the cofactor NAD/NADH which is expensive, hydrolytically unstable and sensitive to oxygen and to light.

A commercial process for the resolution of D,L-amino acids currently exists (see T. Tosa, Biotech. Bioeng. 9, 603, (1967); I. Chibata et al., Methods. Enzymol., 44, 746 (1976)). In the process, the D,L-amino acid is reacted with acetic anhydride to produce the D,L-N-acyl-amino acid. Reaction with the enzyme L-amino acid acylase from porcine kidney or the fungus

Aspergillus oryzae specifically hydrolyzes only the L-N-acyl amino acid, producing a 1:1 mixture of the desired L-amino acid and unreacted D-N-acyl amino acid. These products are separated and the unreacted D-N-racyl amino acid is racemized and recycled through the process. Starting with the N-acyl amino acid, the process requires three discrete steps: enzyme catalyzed hydrolysis of the L-N-acyl amino acid, physical separation of the L-amino acid from the D-N-acyl amino acid and racemization of the D-N-acyl amino acid for recycling through the process.

Summary of the Invention

The present invention provides an enzymatic process capable of producing many alpha amino acids from the readily available L-aspartic acid (or L-aspartate). In the process of the present invention, L-aspartic acid and an alpha-keto acid are reacted in the presence of a transaminase to form L-amino acid and oxaloacetate, followed by decarboxylation of said oxaloacetate to form pyruvic acid. The decarboxylation of oxaloacetate drives the reaction to completion. Yields of 95% or more of the desired L-amino acid are readily obtained. The by-product, pyruvic acid, is readily separated from the L-amino acid and is highly marketable.

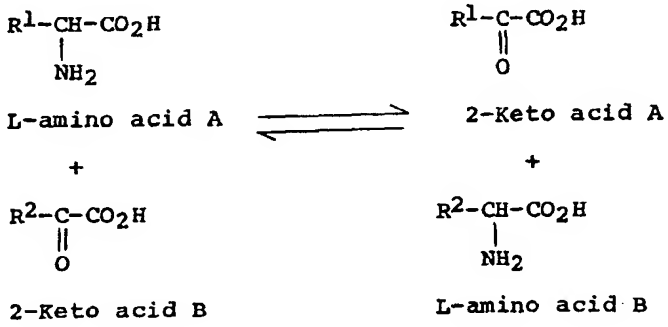
The invention also provides a method for converting D,L-amino acids into L-amino acids in a single step. In the process of the present invention, an enzyme D-amino acid oxidase, is used to convert the D-amino acid in the D,L-mixtures to the

corresponding 2-keto acid. The 2-keto acid is in turn transaminated enzymatically to the corresponding L-amino acid in the same mixture. The two-enzyme catalyzed reactions occur in a single process step, thus producing L-amino acids directly and in high yield from D,L-amino acids.

The conversion of D-amino acids to 2-keto acids using D-amino acid oxidase has been described by Nilsson et al. in Appl. Biochem. Biotechnol., 7, 47-9 (1982). See also Brodelius et al., Appl. Biochem. Biotechnol. 6, 293-308 (1981) and Fink et al., AIChE Symp. Ser. 74, 18-24 (1978). However, there has been no suggestion for using D-amino acid oxidase coupled with a transaminase to convert D,L-amino acid mixtures directly to L-amino acids.

Detailed Description of the Invention

In accord with this invention a class of enzymes known as transaminases (aminotransferases) catalyze the general reaction:



By choosing the proper 2-keto acid precursor B, a desired L-amino acid B can be produced by transamination using another L-amino acid A as the amino donor. As a byproduct of the reaction, a second 2-keto acid A is produced along with the desired L-amino acid B. The advantages of this transamination technology are:

1. L-amino acids are produced specifically.

2. The 2-keto acid precursors are conveniently available from chemical synthesis.

3. The rates of reaction are relatively rapid.

4. The capital costs are lower than for a fermentation process.

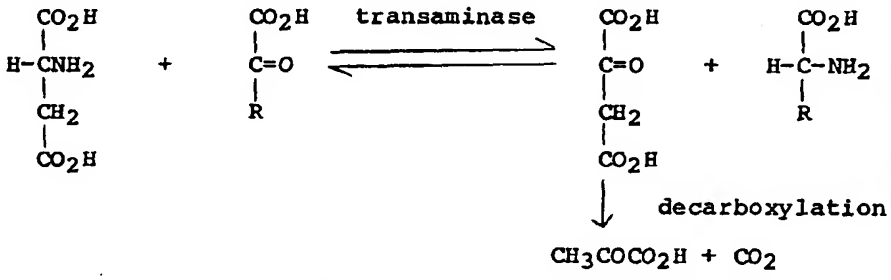
5. The technology is general because transaminases with varying selectivities are available, e.g. aromatic amino acid transaminases, branched chain amino acid transaminases, transaminases specific for amino acids having acidic side chains, etc. Such transaminases can be prepared, for example, from the following microorganisms: Escherichia coli (E. coli), Bacillus subtilis, Achromobacter eurydice, Klebsiella aerogenes, and the like. Transaminases useful in the practice of this invention are also described by H.E. Umbarger in Annual Rev. Biochem., Vol. 47, pp. 533-606 (1978).

The single greatest disadvantage of this general method is that the equilibrium constant for the transamination reaction as written above is about 1.0. As a result, the yield of the desired amino acid for the reaction as written will never exceed

approximately 50%. The key to the development of a commercially successful transamination process for the production of amino acids is overcoming the problem of incomplete conversion of 2-keto acid B to the desired L-amino acid B.

This problem is solved by the present invention by using L-aspartic acid as the amino donor (L-amino acid A) and by converting the byproduct (2-keto acid A), i.e. oxaloacetate, by an irreversible reaction, decarboxylation, to pyruvic acid.

Preferably, the irreversible decarboxylation of oxaloacetate is coupled to the transamination reaction. Thus, the transamination reaction is driven to completion, as shown below:



By coupling the decarboxylation of the oxaloacetate to the transamination reaction in accord with this invention, the production of L-amino acids in high yield can be obtained by this biocatalytic method. Using this method, the conversion of the 2-ketoacid precursor B to the desired L-amino acid B in yields approaching 100% have been achieved.

The decarboxylation of oxaloacetate can be catalyzed either thermally; chemically by various metal ions, amines and/or

acids; or preferably enzymatically by the enzyme oxaloacetate decarboxylase (OAD) E.C. 4:1.1.3. Oxaloacetate decarboxylase from any source can be used. Examples of sources of oxaloacetate decarboxylase useful in the practice of the present invention are, for instance, Micrococcus luteus, renamed from Micrococcus lysodeikticus (see Methods in Enzymology 1, 753-7 (1955) which is incorporated by reference, Pseudomonas putida (see Biochem. Biophys. Acta 89, 381-3 (1964) which is hereby incorporated by reference), and Azotobacter vinelandii (see J. Biol. Chem. 180, 13 (1949) which is hereby incorporated by reference), etc. Also, any other enzyme having an oxaloacetate decarboxylase activity but not usually regarded as an "oxaloacetate decarboxylase" may be used such as, for instance, pyruvate kinase, malic enzyme, etc. The activity of oxaloacetate decarboxylase can be enhanced by adding metal ions such as, for example, Mn^{++}, Cd^{++}, Co^{++}, Mg^{++}, Ni^{++}, Zn^{++}, Fe^{++}, Ca^{++} and the like.

The process of this invention can thus be used for the production of a large variety of L-amino acids by choosing the proper 2-keto acid precursor and an enzyme capable of transaminating it with L-aspartic acid. For example, the amino acid L-phenylalanine, a key component in the manufacture of the sweetener aspartame, has been prepared by this method in high yield from phenylpyruvate and L-aspartic acid using a transaminase isolated from E. coli and an oxaloacetate decarboxylase isolated from either Pseudomanas putida or Micrococcus luteus. Similarly, using these same enzymes,

p-hydroxyphenylpyruvate was converted into L-tyrosine, indole-3-pyruvate or 3-(3-indolyl)pyruvic acid has been converted into L-tryptophan, and 2-oxo-4-methylpentanoic acid has been converted into L-leucine, and 4-phenyl-2-oxobutanoic acid has been converted into L-4-phenyl-2-aminobutanoic acid. By using transaminases with different specificities, 2-oxo-3-methylpentanoic acid was transaminated to L-isoleucine, 2-oxo-3-methylbutanoic acid to L-valine, pyruvic acid to L-alanine, 3-hydroxypyruvate to L-serine, glyoxylic acid to glycine, and 2-oxo-4-thiomethylbutanoic acid to L-methionine.

Thus, R in the keto acid starting material $RCOCO_2H$ can be selected from a wide variety of substituents including, for example, hydrogen, substituted and unsubustituted lower alkyl, substituted and unsubstituted lower aryl, and heterocyclic groups.

The term "lower alkyl" as used herein means both straight and branch chain alkyl groups having from one to about six carbon atoms. Substituted lower alkyl groups means lower alkyl groups substituted with hydroxy, mercapto, carbamoyl, carboxy, amino, amidino and R'-thio (where R' is lower alkyl) groups such as found in natural amino acids.

The term "lower aryl" as used herein means phenyl and benzyl groups. Substituted lower aryl groups includes phenyl and benzyl groups substituted with groups such as those listed above for lower aklyl.

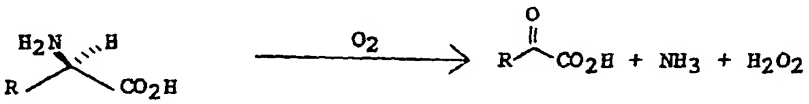
Heterocyclic groups as used herein means

4-imidazoylmethyl, 3 indoylmethyl, and the like.

Examples of such R groups suitable for the practice of the present invention include: hydrogen, methyl, isopropyl, isobutyl, sec-butyl, benzyl, phenyl-1-(methylthio)ethyl, hydroxymethyl, mercaptomethyl, p-hydroxybenzyl, p-hydroxyphenyl, carbamoylmethyl, carbamoylethyl, aminobutyl, amidinoaminopropyl, indolyl, 3-indoylmethyl, imidazoyl, 4-imidazoylmethyl, and the like.

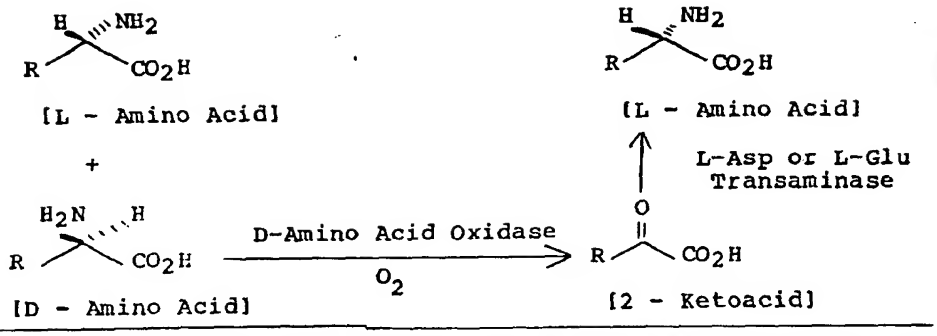
The byproduct of the decarboxylation of oxaloacetate, pyruvic acid, is a valuable commerical product and can be recovered from the product stream by any method described in the prior art, such as acidification and distillation, ion exchange, solvent extraction, and the like.

In accord with another embodiment of this invention a class of enzymes known as D-amino acid oxidases [E.C. 1.4.3.3.1 catalyze the general reaction shown in Scheme 1.



D - Amino Acid 2 - Keto Acid

This enzyme reaction can be coupled to the above transaminase reaction to produce L-amino acids from racemic mixtures of D,L-amino acids in accord with the following reaction scheme:



Molecular oxygen is illustrated in the above reaction scheme as a hydrogen acceptor for D-amino acid oxidase, producing hydrogen peroxide as a byproduct.

However, other hydrogen acceptors such as dyes can also be used.

D-amino acid oxidase can be obtained from any source for use in this invention. Examples include porcine kidney, Trigonopsis variabillis, fungi of the genus Candida, and the like. The D-amino acid oxidase from porcine kidney has been well characterized and an account of the properties of this enzyme can be found in A. Meister and D. Willner, The Enzymes, 1, 634 (1963). Other enzymes useful in the practice of the present invention are those having more substrate specificity such as D-aspartate oxidase (E.C. 1.4.3.1) and D-glutamate oxidase (E.C. 1.4.3.7). Such enzymes will be referred to collectively herein as D-amino acid oxidase unless a more specific enzyme is used. The enzyme from porcine kidney is preferred for use in this invention because it has a broad substrate specificity, acting on all D-amino acids except those with acidic and basic side chains.

It has a specific activity ranging from 20-60 micromoles product produced per minute per milligram of protein, depending on which D-amino acid is chosen as substrate. Oxygen is the most effective election acceptor, but certain dyes can also be used with lower efficiency. The enzyme is not inhibited by L-amino acids or amino acid amines, but is competitively inhibited to varying degrees by 2-keto acids and 2-hydroxyacids.

Appropriate amino group donors for the transamination reaction coupled to the D-amino acid oxidase reaction are L-glutamic acid and L-aspartic acid. Both of these amino acids are readily available and inexpensive. Preferably, L-aspartic acid is used as the amino donor (L-amino acid A) and the byproduct (2-keto acid A), i.e. oxaloacetate is converted by an irreversible reaction, decarboxylation, to pyruvic acid.

Hydrogen peroxide accumulation has been shown to inactivate enzymes (Greenfield et al., Annl. Biochem. 65, 109 (1975)) and can also cause the decomposition of 2-ketoacids. To prolong the half-life of active enzyme in the coupled catalytic system and prevent ketoacid decomposition, the H_2O_2 produced in the step catalyzed by D-amino acid oxidase can be removed by any of several methods for decoupling H_2O_2 well known by those skilled in the art.

One method involves the use of the enzyme catalase, which catalyzes the disproportionation of H_2O_2 to molecular oxygen and water as shown in the reaction:

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$$H_2O_2 \xrightarrow{\text{Catalase}} H_2O + \frac{1}{2} O_2$$

Catalase is commercially available from mammalian liver or Aspergillus niger, with the Aspergillus niger catalase showing better stability. The use of catalases has the added advantage that the disproportionation produces one molecule of O_2 for every two molecules of H_2O_2 generated. Since O_2 is the initial electron acceptor for D-amino acid oxidase, its production by disproportiontion of H_2O_2 by catalase helps supply the oxygen requirement for the oxidation of D-amino acids and partially alleviates the problem of transfer of oxygen from air across a gas-liquid interface. Catalase, however, is slowly inactivated by H_2O_2 as well. A second method for removal of H_2O_2 involves its decomposition by any of several metallic oxides. The use of metal oxides, preferably manganese oxides such as Mn_2O_3, either with or without catalase, offers substantial stability for D-amino acid oxidase, transaminase, and other enzymes used in the practice of this invention.

Thus, the R group in the D,L-amino acid starting material can be selected from a wide variety of substituents including, for example, hydrogen, substituted and unsubstituted lower alkyl, substituted and unsubstituted lower aryl, and heterocyclic groups, which are defined above.

The enzymes can be added to the reaction mixture in whole cells, crude cell lysates, as partially purified enzyme or purified enzyme. Preferably purified enzymes are used, either

immobilized or in solution, because the conversion rates per unit of enzyme are higher. The enzymes can be purified by techniques well known to those skilled in the art. Examples of purification of oxaloacetate decarboxylase from Micrococcus luteus and Pseudomonas putida are described by Herbert, Methods in Enzymology 1, pp. 753-57 (1955) and by Morton et al., Biochem. Biophys. Acta. 89, pp. 381-83 (1964).

The enzymes can be used in solution or as immobilized enzymes, as aforesaid, in the practice of this invention. One example of an immobilized enzyme system is described by Weetall et al., Methods in Enzymology 34, pp. 59-72 (1974), which is hereby incorporated by reference. Weetall et al. describe a method for immobilizing enzymes on glutaraldehyde activated controlled pore glass beads (Corning).

In accord with this method, transaminase was coupled to the glass particles by reacting the enzyme with the activated glass particles at 0-5°C for 2 hours in a phosphate buffer solution having a pH of 7.0. The coupled enzyme can be used directly or first reacted with 1% sodium borohydride to stabilize the covalent link between the enzyme and the activated glass.

Other suitable substrates for immobilizing enzymes for the practice of this invention include porous ceramic, sepharose, diethylaminoethyl cellulose, and the like. These substances can be activated, if desired, by techniques well known in the art.

The oxaloacetate decarboxylase is either immobilized separately, or first mixed with the transaminase and the mixture

co-immobilized. Glass beads on which the enzymes had been covalently attached by the aforedescribed procedures were suspended in a solution containing 10 mM phenylpyruvate, 10 mM L-aspartic acid, 1 mM $MgCl_2$ or $MnSO_4$, pH adjusted to the range 4.0-10.0 and most preferably between 5.5 and 8.5. When all the phenylpyruvate had been consumed, the solution was filtered away from the glass beads and the products L-phenylalanine and pyruvic acid isolated and purified by conventional methods.

The D-amino acid oxidase and transaminase may also be immobilized separately or first mixed together and co-immobilized as a mixture. In like manner, catalase and/or oxaloacetate decarboxylase may also be either immobilized separately or mixed with the other enzymes and the mixture co-immobilized.

Immobilization supports to which the enzymes have been attached can be suspended in a solution containing D,L-amino acid, an amino donor preferably L-aspartic acid, and pyridoxal phosphate (as a cofactor for the transaminase) with the pH adjusted to the range 2.0 to 12.0 and most preferably between 5.5 and 8.5, and the solution incubated at a temperature between 4°C and 50°C and most preferably between 15°C and 40°C. The reaction can be monitored either by polarimetry or by measuring the consumption of L-aspartic acid. When the optical rotation of the solution has ceased changing or no further consumption of L-aspartic acid is noted, the solution is filtered away from the immobilized enzyme and the L-amino acid is isolated and purified by any methods in the prior art such as precipitation.

crystallization, ion exchange chromatography, and the like.

The reaction of L-aspartic acid to produce L-amino acids and pyruvic acid can be monitored if desired. A general assay which is applicable to the assay of all transamination reactions using L-aspartic acid as the amino donor regardless of the 2-keto acid precursor that is used is the following: L-aspartic acid, a 2-keto acid, transaminase, NADH, and the enzyme malic dehyrogenase (available commercially) are dissolved in solution of phosphate buffer as a pH between 6.0 and 9.0; the change in the absorbance at 340 nm (A_{340}) with time is measured. This change in the absorbance at 340 nm corresponds to the consumption of NADH during the reduction of oxaloacetate, formed from L-asparate during the transamination reaction.

As an alternative, for instance, the conversion of phenylpyruvate to L-phenylalanine can be conveniently assayed by taking aliquots from the reaction mixture containing, for instance, transaminase, phenylpyruvate, L-aspartate, oxaloacetate decarboxylase, and metal ions, diluting them into a solution of 2.5% sodium hydroxide in water (w/v), and measuring the absorbance at 320 nm. Dilution into sodium hydroxide causes rapid achievement of the equilibrium between the keto and enol forms of phenylpyruvate. The extinction coefficient at 320 nm for the equilibrium mixture is 17500 M^{-1} cm^{-1}. Thus, the conversion of phenylpyruvate into L-phenylalanine can be quantitated rapidly. This asssay can be corroborated by measuring L-phenylalanine qualitatively by paper chromatography

and quantitatively using an amino acid analyzer.

Similar techniques can be used to assay for the conversion of other 2-keto acids into the corresponding L-amino acids. The transamination of p-hydroxyphenylpyruvate to L-tyrosine can be monitored by diluting aliquots removed from the reaction mixture into 2.5% NaOH and measuring the absorbance at 331 nm (extinction coefficient of 19900 m^{-1} cm^{-1}, and the conversion of indole-3-pyruvate into L-tryptophan can likewise be followed by measuring the absorbance at 328 nm (extinction coefficient of 10000 M^{-1} cm^{-1}).

The invention will now be further illustrated by the following examples which are given here for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1 Preparation of Aromatic Acid Transaminase

E. coli K-12 maintained on L-broth plates was innoculated into 2.0 liter shake flasks containing 500 ml of the medium listed below:

KH_2PO_4	5 g/Liter
K_2HPO_4	5.56 g/liter
$(NH_4)_2SO_4$	2 g/liter
$MgSO_4$	75 mg/liter
Na_3 (citrate)$\cdot 2H_2O$	1 g/liter
*Trace Metals	3 ml/liter
Glucose	10 g/liter

*Preparation of Trace Metals Solution

Metal Salts	Amount	Final concentrations
$FeCl_3 \cdot 6H_2O$	27 g/l	300 uM
$ZnCl_2$	1.3 g/l	30 uM
$CoCl_2 \cdot 6H_2O$	2 g/l	25 uM
$Na_2MoO_4 \cdot 2H_2O$	2 g/l	25 uM
$CaCl_2 \cdot 2H_2O$	1 g/l	20 uM
$CuCl_2 \cdot 2H_2O$	1.27 g/l	22 uM
H_3BO_3	0.5 g/l	24 uM
HCl (conc)	100/ml/l	3.6 uM

Growth was at 37 C for 15 hours. These flasks were used to innoculate 14 liter Biolafitte fermenters (1 liter of shake flask culture into 7 liters) containing 7 liters of the growth medium listed below:

KH_2PO_4	2.0 g/liter
K_2HPO_4	3.6 g/liter
$(NH_4)_2SO_4$	750 mg/liter
Na_3(citrate)$\cdot 2H_2O$	1 g/liter
Trace metals	3 ml/liter
Pump in glucose as needed.	

Growth was at 37°C with aeration at 300 rpm and the pH was maintained at 6.9 by titration with ammonium hydroxide. The cells were harvested by centrifugation at 4000 rpm and frozen at -10°C until needed.

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Purification of the Aromotic Acid Transaminase

All steps were carried out at 4°C. Centrifugations were carried out in a Sorvall RC2B centrifuge.

1. E. coli K-12 cells (80g wet weight) were resuspended in 200 ml of an aqueous buffer solution, pH 7.0, containing 200 mM potassium phosphate, 1mM ethylenediaminetetraaccetic acid (EDTA) disodium salt, 1mM beta-mercaptoethanol, 1mM pyridoxal phosphate, and 0.02% (weight/volume) sodium azide. The cells were sonicated using a Heat Systems - Ultrasonics Cell Disruptor with 4 one minute bursts, power setting 9. The cell debris was separated by centrifugation at 12,000 rpm for 20 minutes.

2. The crude extract (supernatant from step 1) was made 1.25% weight/volume in streptomycin sulfate by adding the appropriate amount of a 40% streptomycin sulfate solution prepared in the buffer of step 1. The mixture was stirred slowly for 20 minutes then centrifuged at 12,000 rpm for 20 minutes. The precipitate was discarded.

3. The protein in the supernatant from step 2 was fractioned by the addition of ammonium sulfate. Crystalline ammonium sulfate was added with stirring until a concentration 40% of saturation was attained and the protein precipitate was centrifuged and discarded. Additional ammonium sulfate was added with stirring until a concentration 70% of saturation was attained and the protein precipitate was centrifuged, collected,

and redissolved in the minimum amount of a buffer, pH 6.5, containing 0.03 M sodium phosphate, 1mM ethylenediaminetretraacetic acid disodium salt, 1mM beta-mercaptoethanol, and 0.02% (weight/volume) sodium azide. This solution was dialyzed against 2 liters, of the same buffer (18 hours, 2 changes of buffer).

4. A DEAE-cellulose column (Whatman DE-52, 1.6 x 30 cm) was equilibrated with the buffer from step 3. The sample was loaded on the column and washed until no more protein could be detected in the effluent as measured by the OD_{280} (<0.02). A 0 - 0.5 M NaCl linear gradient was established, 250 ml total volume, flow rate = 4 ml/10 minutes/fraction. Transaminase activity eluted between 0.09 and 0.2 M NaCl and was pooled and dialyzed against 2 x 2 liters of a buffer, pH 6.5, containing 0.03 M sodium phosphate, 1mM ethylenediaminetetraacetic acid disodium salt, 1mM beta-mercaptoethanol, 0.02 mM pyridoxal phosphate.

5. The transaminase solution was loaded onto a column of hydroxyapatite (2.6 x 30 cm) and equilibrated in the dialysis buffer of step 4. The transaminase activity was not retained by the column and was concentrated to approximately 4 ml using an Amicon ultrafiltration apparatus with a YM 30 membrane.

6. The concentrated transaminase from the previous step was loaded onto a Sephacryl S-200 column, 2.6 x 90 cm, in a solution of 0.05 M Tris pH 8.0, 0.02 mM pyridoxal phosphate, 1mM ETDA, and 1mM beta-mercaptoethanol. Elution with the same buffer gave a band of transaminase activity eluting soon after the void

volume. This material was stored at 4°C and was stable for at least 4 months.

Oxaloacetate decarboxylase can be prepared from Micrococcus luteus, Pseudomonas putida, or the like, by similar procedures as is well known in the art.

Example 2. Preparation of L-Phenylalanine

To 0.8ml of pH 7.0 solution consisting of 50mM potasium phosphate buffer, 12.5mM phenylpyruvate, 25mM L-aspartic acid, 1.25mM manganese sulfate, 5mM pyridoxal phosphate, and 1.5 international units of oxaloacetate decarboxylase was added 0.2ml of a solution at pH 7.0 containing 0.3 international unit (IU) of transaminase. Both immediately, and after incubation at 22°C for 12 hours, the reaction mixture was assayed for phenylpyruvate. The level of conversion was calculated to be 98.5% based on the amount of phenylpyruvate converted. Amino acid analysis of the reaction mixture showed only two peaks corresponding to L-phenylalanine and unreacted L-aspartic acid. No other amino acid products were detected.

Example 3. Immobilization of transaminase and oxaloacetate decarboxylase

An aqueous solution of 2.0ml of 50mM potassium phosphate, pH 8.0, containing 1.5 units of transaminase and 5.4 units of oxaloacetate decarboxylase isolated from Micrococcus luteus was added to 5ml of DEAE-celulose gel (Whatman DE-52) previously equilibrated to pH 8.0 in 50mM phosphate buffer. After gentle

agitation for 5 minutes, assaying for both the transaminase and the oxaloacetate decarboxylase indicated that the enzymes had been adsorbed on the DEAE-celulose and the remaining solution was decanted from the gel.

Example 4. Alternative Preparation of L-Phenylalanine

To the immobilized enzyme preparation of Example 3 were added solutions of potassium phosphate, 50 mM, pH 8.0, 2.0 ml; phenylpyruvate,50mM,0.5ml; l-aspartate, 50mM, 0.5 ml, and manganese sulfate, 10 mM, 1.0 ml. After a 12 hour incubation, the assay indicated a level of conversion of phenylpyruvate to L-phenylalanine of 96%. This was confirmed by the detection of L-phenylalanine by paper chromatography using an elution solvent of n-butanol:acetone:ammonium hydroxide:water 5:3:1:1. Determination of the amino acid content of the reaction mixture using a Dionex amino acid analyzer showed only two detectable peaks corresponding to L-phenylalanine and L-aspartic acid.

The L-phenylalanine was purified using BioRad AG 50 1X8 20-50 mesh mixed bed ion exchange resin. The crude reaction mixture at pH8.0 was passed down a column of the resin previously eguilibrated to the same pH and the column was eluted first with 2 column volumes of water and then 50 mM potassium phosphate buffer, Ph 8.0. Phenylalanine can be recovered from the eluent by lyophilization or by acidification and crystallization.

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Example 5. Alternative Preparation of L-Phenylalanine

A 19 ml solution of 50mM potassium phosphate containing 0.5mM pyridoxal phosphate, 1mM $MnSO_4$, 5 international units of transaminase isolated from E. coli, 10 international units of oxaloacetate decarboxylase isolated from Micrococcus luteus, 50 mM phenylpyruvate, and 65 mM L-aspartic acid was incubated at 12 hours at 24°C. At the end of this time, quantitation of the amount of phenylpyruvate by removing a 50 microliter aliquot, diluting to 1.0ml with 2.5% NaOH, and reading the optical density at 320 nm indicated that all of the phenylpyruvate had been converted to L-phenlalanine. Paper chromatography on Whatman 3MM paper using butanol:acetone:ammonium hydroxide:water 5:3:1:1 as eluent, followed by staining with 5% ninhydrin dissolved in acetone, showed only two ninhydrin active spots corresponding to unreacted L-aspartic acid and to L-phenylalanine.

Example 6. Preparation of L-Tyrosine

A solution buffered at pH 7.0 by 50mM potassium phosphate containing $MgSO_4$, 1mM; p-hydroxyphenylpyruvate, 10mM; L-aspartic acid, 10mM; transaminase, 0.1 mg/ml; and oxaloacetate decarboxylase isolated from Pseudomonas putida (ATCC 950), 0.1 mg/ml was incubated for 1 hour at 24°C. At the end of this time, assay indicated that no p-hydroxyphenylpyruvate remained. Quantitation of the amount of L-tyrosine produced by the injection of an aliquot in a Dionex amino acid analyzer gave a yield of 99% on a molar basis from p-hydroxyphenylpyruvate.

The L-tyrosine and the pyruvic acid so produced can be purified by a method similar to that used for L-phenylalanine in Example 4 or other methods well known in the art.

Example 7. Preparation of L-Tryptophan

A solution of indole-3-pyruvate, 20mM; L-aspartic acid 20mM; $MgCl_2$, 1.5mM; transaminase, 0.3 mg/ml; oxaloacetate decarboxylase, 0.3 mg/ml buffered to pH 7.0 with 5mM tris-hydroxymethylaminomethane hydrochloride (Tris) was stirred slowly for 2 hours. At the end of this time the reaction is complete. The L-tryptophan and pyruvic acid produced can be purified by methods well known in the art.

Example 8. Preparation of L-Leucine

A solution containing $MgCl_2$, 2.5 mM; -2-oxo-4-methylpentanoic acid, 100 mM; L-aspartic acid, 100 mM; transaminase, 1.0 mg/ml; oxaloacetate decarboxylase, 1.0 mg/ml, pH adjusted to 7.0 with NaOH, was stirred slowly at 30°C for 4 hours. The L-leucine and the pyruvic acid formed can be purified by any methods well known in the art.

Example 9. Preparation of L-Valine

A solution containing $MgCl_2$, 2.5 mM; 2-oxo-3-methylbutanoic acid, 100 mM; L-aspartic acid, 100 mM; transaminase, 1.0 mg/ml; oxaloacetate decarboxylase, 1.0 mg/ml, pH adjusted to 7.0 with NaOH, is stirred slowly at 30°C for 4

hours. The L-valine and the pyruvic acid formed can be purified by any methods well known in the art.

Example 10. Preparation of L-Serine

A solution containing $MgCl_2$, 2.5 mM; 3-hydroxypyruvate, 100 mM; L-aspartic acid, 100 mM; transaminase, 1.0 mg/ml; oxaloacetate decarboxylase, 1.0 mg/ml, pH adjusted to 7.0 with NaOH is stirred slowly at 30°C for 4 hours. The L-serine and the pyruvic acid formed can be purified by any methods well known in the art.

Example 11. Preparation of L-Methionine

A solution containing $MgCl_2$, 2.5 mM;-2-oxo-4-thiomethylbutanoic acid, 100 mM; L-aspartic acid, 100 mM; transaminase, 1.0 mg/ml; oxaloacetate decarboxylase, 1.0 mg/ml, pH adjusted to 7.0 with NaOH, is stirred slowly at 30°C for 4 hours. The L-methionine and the pyruvic acid formed can be purified by any methods well known in the art.

Example 12. Preparation of L-4-phenyl-2-aminobutanoic acid by E. coli K-12 Transaminase

Ethyl 4-phenyl-2-oxabutanoate (Chemical Dynamics) (206 mg, 1 mMole) was suspended in 10 milliliters of 0.1M NaOM, and the mixture was stirred for 30 minutes until the pH dropped to below 8.6, indicating complete hydrolysis of the ethyl ester. The mixture was centrifuged briefly, and the colorless supertanant containing 4-phenyl-2-oxobutanoic acid sodium salt was decanted.

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A solution containing 0.700 ml pH 8.0 50mM potassium phosphate buffer, 0.10 ml of the above 4-phenyl-2-oxobutanoate solution, 0.050 ml, 500mM disodium L-aspartate, 1.0 international unit of malic dehydrogenase, 0.1 international units of aspartic transaminase purified from E. coli K-12, 0.1 micromole pyridoxal phosphate (Sigma), and 0.25 mg nicotinamide adenine dinucleotide in the reduced form (Sigma) was prepared. The decrease in the absorbance of the solution at 360 nm was indicative of reaction. This change was not observed in controls in which transaminase, malic dehydrogenase, L-aspartate, or 4-phenyl-2-oxobutanoate were the only component omitted. The rate of the reaction was approximately 16-18% of the corresponding reaction using phenylpyruvate in place of 4-phenyl-2-oxobutanoate.

Example 13. Production of L-4-Phenyl-2-Aminobutanoic Acid By Immobilized Enzymes

Transaminase from E. coli K-12 and oxaloacetate decarboxylase from Pseudomonas putida ATCC 950 are immobilized on succinyl aminopropyl porous glass (Corning) by covalent attachment using the carbodiimide method described by Weetall. The immobilized enzyme is loaded into a glass column. A solution of 2-keto-4-phenylbutanoic acid (18 g/liter), L-aspartic acid (15 g/liter), pyridoxal phosphate (0.05 g/liter), $MgO_2 \cdot 6H_2O$ (2.03 g/liter) buffered by 50mm potassium phosphate with a pH of 7.1 is pumped through the column and the effluent is collected in fractions. The extent of conversion is monitored by assaying for pyruvic acid using lactic dehydrogenase. The

L-4-phenyl-2-aminobutanoic acid and pyruvic acid products are purified by ion exchange chromatography to yield pure L-4-phenyl-2-aminobutanoic ($[\alpha]_D^{20} = -47°$) and pyruvic acid.

Example 14. Preparation Of L-Phenylalanine From A D,L Racemic Mixture

D,L-phenylalanine (1.65 g/liter), pyridoxal phosphate (0.028 g/liter), transaminase from E. coli (2,500 units/liter), D-amino acid oxidase from porcine kidney (1,000 units/liter), oxaloacetate dicarboxylase from Psuedomonas putida (ATCC 950; 20,000 units/liter), and catalase from Aspergillus niger (greater than 100,000 units/liter), were mixed at a pH of 7.0 in a shallow cylindrical vessel by rotary mixing for 80 minutes. Aliquots were taken at regular intervals and assayed for pyruvate produced as a message of percentage conversion.

The rate of conversion of D,L-phenylalanine to L-phenylalanine was linear over the period 0-80 minutes. Based on this rate, the productivity of the catalytic system was 1.86 mmoles D-phenylalanine in the D,L-mixture converted to L-phenylalanine per hour.

Example 15. Alternative Preparation Of L-Phenylalanine From A D,L Racemic Mixture

D,L-phenylalanine (1.42 g/liter), L-aspartic acid (1.33 g/liter), pyridoxal phosphate (0.056 g/liter), $MgCl_2.6\ H_2O$ (2.03 g/liter) D-amino acid oxidase from porcine kidney (125 units/ml), catalase from bovine liver (greater than 100,000 units/ml), transaminase

from E. coli K-12 (5,000 units/liter), oxaloacetate decarboxylase from Pseudomonas putida (ATCC 950; 19,000 units/liter) were incubated at pH 7.5 by rotary shaking in a shallow cylindrical vessel. Aliquots were taken at time intervals and assayed for pyruvate as a measure of completeness of the reaction. After 105 minutes, 3.51 mmoles pyruvate had been produced per liter for a conversion of 81%. The productivity of the catalytic system under these conditions was 346mg L-phenylalanine produced from the D-phenylalanine in the D,L-mixture per hour.

Example 16. Preparation Of L-Leucine From A D,L Racemic Mixture

D,L-leucine (13.2 g/liter), L-aspartic acid (0.68 g/liter), pyridoxal phosphate (0.070 g/liter), $MgCl_2 \cdot 6\ H_2O$ (2.03 g/liter), D-amino acid oxidase from porcine kidney (15,000 units/liter), catalase from Aspergillus niger (150,000 units/liter), transaminase from E. coli K-12 (8,000 units/liter), oxaloacetate decarboxylase from Pseudomonas putida ATCC 950 (20,000 units/liter) are incubated at 25°C and at pH 7.5 with rotary shaking in a cylindrical vessel with baffler to increase oxygenation of the solution. The reaction is followed to completion by removing aliquots and assaying for pyruvate. L-leucine is purified from the reaction mixture by ion exchange chromatography to yield a pure product. Pyruvic acid is also recovered similarly as its sodium salt.

Example 17. Preparation Of L-Valine From A D,L Racemic Mixture

D,L-Valine (23.4 g/liter), L-aspartic acid (13.3 g/liter), pyridoxal phosphate (0.060 g/liter), $MgCl_2 \cdot 6H_2O$ (2.03 g/liter) D-amino acid oxidase (15,000 units/liter), catalase (150,000 units/liter), E. coli transaminase B, E.C. 2.6.1.6, see Monnier et al., Biochemie (1976) 58, 663-675, (10,000 units/liter), oxaloacetate decarboxylase from Pseudomonas putida ATCC 950 (25,000 units/liter) are incubated with shaking and aeration at 25°C and pH 7.5. The products L-valine and pyruvic acid are separated and purified by ion exchange methods to yield homogeneous L-valine and homogeneous sodium pyruvate.

The invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of the disclosure herein, may make modifications and improvements within the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A process for producing alpha amino acids or derivatives thereof, said process comprising reacting an alpha-keto acid with L-aspartic acid in the presence of transaminase enzyme to produce (1) an alpha amino acid corresponding to said alpha-keto acid and (2) oxaloacetate; and decarboxylating said oxaloacetate.

2. The process of claim 1 wherein the said transaminase is a purified or partially purified enzyme preparation, or is contained within a whole cell.

3. The process of claim 1 wherein the said step of decarboxylating oxaloacetate is accomplished using an oxaloacetate decarboxylase enzyme.

4. The process of claim 3 where the said oxaloacetate decarboxylase enzyme is a purified or partially purified enzyme preparation, or is contained within a whole cell.

5. The process of claim 3 wherein said transaminase and said oxaloacetate decarboxylase are each immobilized on an insoluble support.

6. The process of claim 5 wherein said immobilization support is controlled pore ceramic particle or controlled pore glass particle.

7. The process of claim 5 wherein said transaminase and said oxaloacetate decarboxylase are both immobilized on the same support.

8. The process of claim 5 wherein the said transaminase and oxaloacetate decarboxylase are adsorbed on diethylaminoethyl cellulose.

9. The process of claim 1 wherein said transaminase is an aromatic amino acid transaminase.

10. The process of claim 1 wherein said alpha-keto acid is selected from the group consisting of phenylpyruvic acid, p-hyroxyphenylpyruvic acid, 3-(3-indolyl)pyruvic acid, 3-(4-imidazoyl)pyruvic acid.

11. A process in accord with claim 5 wherein phenylpyruvic acid is reacted with L-aspartic acid in the presence of an aromatic amino acid transaminase and an oxaloacetate decarboxylase, thus producing L-phenylalanine.

12. The process of claim 1 wherein the said transaminase is an enzyme selective for the transamination of amino acids with branched side-chains.

13. The process of claim 1 wherein the said alpha-keto acid is selected from the group consisting of 2-oxo-3-methylbutanoic acid, 2-oxo-4-methylpentanoic acid, 2-oxo-3-methylpentanoic acid, and 3-hydroxypyruvic acid.

14. The process of claim 1 wherein the said transaminase is an enzyme capable of catalyzing the transamination of hydroxypyruvate to L-serine.

15. The process of claim 1 wherein the said transaminase is an enzyme isolated from a microorganism selected from the group consisting of Escherichia coli, Bacillus subtilis, Achromobacter eurydice, or Klebsiella aerogenes.

16. The process of claim 3 wherein the said oxaloacetate decarboxylase is an enzyme isolated from a microorganism selected from the group consisting of Micrococcus luteus, Pseudomonas putida, or Azotobacter vinelandii.

17. The process of claim 3 wherein the said oxaloacetate decarboxylase is reacted in the presence of a metal ion selected from the group consisting of Mn^{++}, Cd^{++}, Co^{++}, Mg^{++}, Ni^{++}, Zn^{++}, Fe^{++}, and Ca^{++}.

18. A process for producing alpha L-4-phenyl-2-aminobutanoic acid or derivatives thereof, said process comprising reacting 4-phenyl-2-oxobutanoic acid with L-aspartic acid in the presence of transaminase enzyme to produce L-4-phenyl-2-aminobutanoic acid and oxaloacetate, and decarboxylating said oxaloacetate.

19. A process for producing L-amino acids directly from the corresponding D,L-amino acid racemic mixture, said process comprising reacting said D,L-amino acid mixture in admixture with a D-amino acid oxidase and a transaminase in the presence of a suitable amino group donor.

20. The process of claim 19 wherein said amino group donor is L-glutamic acid.

21. The process of claim 19 wherein said amino group donor is L-aspartic acid.

22. The process of claim 21 wherein said admixture further comprises an oxaloacetate decarboxylase.

23. The process of claim 19 wherein said admixture further comprises a catalase.

24. The process of claim 23 wherein said admixture contains a metal oxide.

25. The process of claim 24 wherein said metal oxide is a manganese oxide.

26. The process of claim 25 wherein the said manganese oxide is Mn_2O_3.

27. The process of clam 19 wherein the D-amino acid oxidase and transaminase are each a purified or partially purified enzyme preparation, or are contained within whole cells.

28. The process of claim 27 wherein the enzymes are immobilized on an insoluble support.

29. A process of claim 28 wherein said immobilization support is controlled pore ceramic particle or controlled pore glass particle.

30. The process of claim 28 wherein the said enzymes are immobilized on the same support.

31. The process of claim 28 wherein the said enzymes are adsorbed on diethylaminoethyl cellulose.

32. The process of claim 19 wherein said transaminase is an aromatic amino acid transaminase.

33. The process of claim 19 wherein the said transaminase is an enzyme isolted from a microorganism selected from the group consisting of Escherichia coli, Bacillus subtilis, Achromobacter eurydice, or Klebsiella aerogenes.

34. The process of claim 22 wherein the said oxaloacetate decarboxylase is an enzyme isolated from a microorganism selected from the group consisting of Micrococcus luteus, Pseudomonas putida, or Azotobacter vinelandii.

35. The process of claim 22 wherein the said oxaloacetate decarboxylase is reacted in the presence of a metal ion selected from the group consisting of Mn^{++}, Cd^{++}, Co^{++}, Mg^{++}, Ni^{++}, Zn^{++}, Fe^{++}, and Ca^{++}.